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CHARACTERIZATION OF MUSTARD-STIMULATED PROTEASE BY ZYMOGRAPHY

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ABSTRACT

Protease stimulation in human epidermal keratinocytes (HEK) due to mustard exposure is well established. However, the specific protease(s) stimulated by mustard remain to be determined. We used zymography technique to characterize the mustard-stimulated protease in HEK extracts. The major proteolytic band in 70kDa molecular weight was observed in mustard-exposed cells. Addition of a serine protease inhibitor (ICD 2812) or EGTA decreased this band. The protease from mustard treated HEK was electro eluted from the zymogram gel and was found to hydrolyze the synthetic substrate Chromozyme TRY, supporting that it could be a calcium dependent serine-protease.

INTRODUCTION

Protease stimulation in epidermal keratinocytes is one of the mechanisms of mustard-induced vesication. Mustard stimulated proteases cause the separation of the epidermis from the dermis by degrading attachment proteins such as laminin-5. The purpose of this research was to establish new technologies and to obtain new knowledge required to identify the specific mustard stimulated protease(s), its functions, and inhibitors. The use of protease inhibitors is one of the several pharmacological antivesicant approaches currently under consideration in the advanced vesicant countermeasure DTO. In this context, experiments done at the USAMRICD led to two critical observations (a) in the mini pig skin, which is more akin to the human skin, only one protein in the lamina lucida area i.e., laminin is affected by HD(1), and (b) in human skin explants, laminin-5 immunoreactivity is decreased by HD (2). A defect in laminin-5 subunit composition, especially in b3 and g2, has been implicated in a human blistering disease at the level of lamina lucida (3). These findings strongly suggest that some specific protease(s) may be responsible for HD-induced vesication involving laminin-5 degradation. This concept of a specific protease being involved in HD pathology is important because the use of generalized protease inhibitors in preventing HD toxicity may be contraindicated. We purified and

partially characterized a single protease that hydrolyzes laminin *in vitro* (4). Here we present our observations on how the Zymogram technology may be useful to monitor protease changes in mustard-exposed cells.

METHODS

Normal human epidermal keratinocytes (NHEK) were used in this study. Cells were exposed to HD at USAMRICD according to their approved protocol. Cell lysates were prepared at 16 hours later and in Mammalian Protein Extraction reagent from PIERCE. Protein concentration was determined by the BCA™ protein assay from PIERCE. Equal amounts of lysate proteins from untreated and mustard treated samples were loaded for analysis. Gelatinase zymography was conducted by electrophoresis in polyacrylamide gels containing sodium dodecyl sulfate (SDS) and gelatin (invitrogen) as described by Heussen and Dowdle (Anal Biochem., 102:196, 1980). The protease band was cut and electro-eluted by Bio-Rad electro-eluter (Model 422). The activity of this band was measured by the chromozym TRY method (Chakrabarti, AK. And Ray, P., Biochem. Pharma. 56:467-472, 1998). Laminin-5 and its γ2 chain was detected by Western blot analysis using a monoclonal antibody (Chemicon) against Laminin-5 γ2 following the supplier's instructions.

RESULTS

First, we studied the effects of sulfur mustard on normal human epidermal keratinocytes (NHEK). NHEK were exposed to 300μM HD and cells lysates were prepared at 16 hours later. White lytic zones represent gelatinolytic activity. Gelatin zymograms of untreated NHEK demonstrated a major 72 kDa and two minor 70 and 64 kDa bands (*Fig. 1*). The 72 kDa band increased in HD-exposed cells. The 72 kDa and 70 kDa bands were cut and electro eluted. The 72 kDa band showed increased HD stimulated protease activity by the Chromozym TRY method compared to untreated control (*Fig. 2*). Addition of a serine protease inhibitor ICD 2812 (50 μM) to NHEK prior to HD exposure decreased the HD stimulated 72 kDa band (*Fig. 3*).

Figure 1.

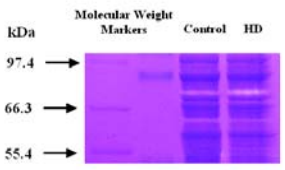
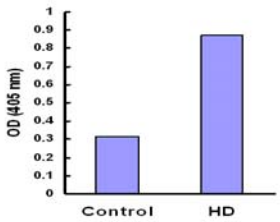
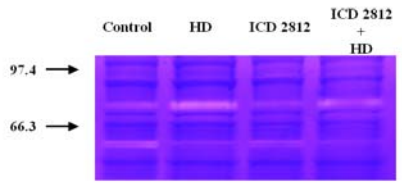


Figure 2.



Next, we investigated the effects of HN_2 on NHEK. NHEK were exposed to HN_2 (300 μM) for the indicated times and then analyzed for protease stimulation by gelatin zymography. The 64 kDa band increased with the time of HN_2 exposure (*Fig. 4*). Addition of EGTA one hour before HN_2 treatment (*Fig. 5*) inhibited both protease stimulation (Zymography) above and laminin-5 γ^2 decrease (Western Blotting) below due to HN_2.

Figure 3.



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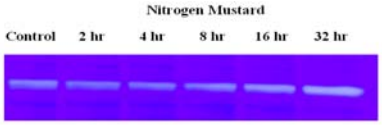

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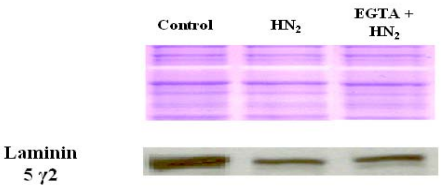

CONCLUSION

Protease stimulation due to sulfur mustard (HD) and nitrogen mustard (HN_2) was analyzed by gelatin zymography. HD (300 μM, 16 hr) stimulated an approximately 72 kDa band containing serine protease activity. HN_2 (300 μM, 16 hr) stimulated an approximately 64 kDa band containing serine protease activity in a time-dependant manner. The HD stimulation of the protease band was inhibited by a serine protease inhibitor (ICD 2812, 50 μM). The HN_2 stimulation of the protease was sensitive to a Ca^{2+} chelator (EGTA, 2 mM) and appeared to hydrolyze laminin-5. We conclude that in NHEK, both HD and HN_2 stimulated an apparently 64 to 72 kDa Ca^{2+} sensitive serine protease that hydrolyzes laminin-5.

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